It was hypothesized that hyperbaric oxygen (HBO) could increase bone healing efficiency according to a protocol with a special window of healing time when maxillary sinus lateral augmentation is performed with only xenograft. The histomorphometric efficiency of HBO on the maxillary sinus lateral augmentation was examined by designing five different in vivo healing periods. Five patients receiving maxillary sinus lateral augmentation with xenograft each received a different treatment healing protocol: 6 weeks natural healing (control [Ctl]), 5 weeks with HBO (T1), 6 weeks with HBO (T2), 9 weeks with HBO (T3), and 13 months natural healing (TM). Biopsy samples were harvested, and quantitative histomorphometric analysis was performed regarding key factors BMP-2 and RUNX2. Analysis of variance and Tukey test were used for pairwise comparisons. Time-dependent relationships of the factors’ expression densities were conducted using quadratic regression fitting. There were statistically significant differences among the groups, except for T2/T3 and T2/TM for BMP-2 and for T2/T3 and TM/Ctl for RUNX2. Both BMP-2 and RUNX2 showed quadratic trends, presenting an initial upward trend and eventually a downward trend depending on T1, T2, and T3 groups. Early stimulation, achieved by keeping HBO until 6 to 9 weeks after maxillary sinus lateral augmentation with xenograft, seemed to be the time window that benefitted bone healing efficiency the most. Int J Periodontics Restorative Dent 2021;41:e81–e91. doi: 10.11607/prd.5477
tension), which can in turn increase the amount of oxygen delivered to hypoxic sites.\(^5\) This may stimulate cellular proliferation and collagen synthesis with positive effects on healing.\(^6\) HBO is used to improve wound repair, as it stimulates oxygenation, cell proliferation, and neovascularization, positively resulting in osteogenesis.\(^7\)

Although HBO was considered a valuable adjunct for the treatment of bone defects and is an established modality in the treatment of many disorders together with successful treatments in clinical conditions related to ischemia and/or hypoxia, it is still under investigation.\(^8\) The variations of clinical HBO protocols for both humans and animals make it difficult to compare reported results, limiting the full understanding of HBO’s biologic effects and mechanisms in tissue repair.

Bone healing is a complex process that is influenced by several factors, such as severity of injury, infection, age, health, and nutrition. Bone morphogenetic protein 2 (BMP-2), Runt-related transcription factor 2 (RUNX2), osteocalcin, and vascular endothelial growth factor are four key factors that play important roles in the bone healing process.

The aim of the present in vivo study was to examine the efficiency of HBO after in vivo maxillary sinus lateral augmentation. Each patient was randomly assigned to one of the five windows of healing time, three of which followed the use of HBO protocols: 6 weeks natural healing (control [Ctl] group), 5 weeks with HBO (T1), 6 weeks with HBO (T2), 9 weeks with HBO (T3), and 13 months natural healing (TM group). Histomorphometric and quantitative analyses were investigated regarding BMP-2 and RUNX2 expression intensities with different healing regimes. To the present authors’ knowledge, there are currently no reports regarding the efficacy of HBO on bone regeneration during in vivo maxillary sinus lateral augmentation with xenograft. The null hypothesis was that HBO could increase the bone regenerative efficiency according to a special time-window protocol when maxillary sinus lateral augmentation is performed with only xenograft.

### Material and Methods

All patients were referred to China-Japan Friendship Hospital in Beijing from October 2016 to December 2017 for implant placement requiring maxillary sinus lateral augmentation. Five patients were enrolled in this study (three men and two women) with an average age of 54.8 years (range: 32 to 72 years). All subjects signed informed consents, which were approved by the local ethics committee for human clinical research in China-Japan Friendship Hospital (no. zryyc/2015/27-1). The Declaration of Helsinki standards was followed.

At the initial visit, all patients received clinical and occlusal examinations. Periapical and panoramic radiographs were performed together with CBCT scans to evaluate possible intrasinus pathologies as well as anatomical sinus morphology. Contraindications of HBO therapy were strictly observed, and absolute contraindications included untreated spontaneous mediastinal emphysema, pulmonary bullous, active bleeding and hemorrhagic disease, and tuberculosis hemoptysis.

All surgical sites were located unilaterally in the maxillary molar regions. Upon reentry at the various time points designated for implant placement, a bone core sample was harvested with a trephine prior to implant insertion at the same position.

### Surgical Protocol

Full-thickness, mucoperiosteal flaps were reflected, exposing the lateral wall of the maxillary sinus. Piezosurgical instrumentation was utilized under copious irrigation of physiologic saline solution for outlining a large buccal window on the maxillary sinus lateral wall. Care was taken not to penetrate the sinus membrane. Once the outline was completed, a delicate dissection was performed using blunt sinus curettes to reflect the sinus membrane medially and coronally along the medial antral wall. The sinus membrane was released without tension to provide an adequate compartment for grafting, allowing for around 12 to 14 mm in vertical height from the upper released sinus membrane to the level of the alveolar crest. Xenograft (Bio-Oss, small particle size: 0.25 to 1.0 mm) was then packed into the sinus cavity compartment. The buccal window of the maxillary sinus wall was covered by collagen membrane (Bio-Guide, Geistlich). The mucoperiosteal flap was then...
repositioned. Horizontal mattress sutures combined with interrupted sutures were performed, achieving tension-free primary closure.

Amoxicillin (1 g twice a day) was prescribed for 1 week along with analgesics to be taken as required. Patients were instructed to rinse with chlorhexidine gluconate for 30 seconds, three times a day. Sutures were removed 2 weeks after surgery.

HBO Protocols

One treatment healing protocol (Ctl, T1, T2, T3, and TM) was assigned per patient. T1, T2, and T3 patients started HBO therapy 24 hours after surgery. Under the action of 100% oxygen at twice the atmospheric pressure (2 ATA), it took 30 minutes to raise the pressure of the oxygen cure tank. The pressure was maintained for 60 minutes, then lowered for the following 30 minutes to finish the treatment. The protocol was performed once a day for 10 days; after 10 days, HBO therapy was paused for 5 days. This procedure (10 days with HBO, 5 days without HBO) was repeated until the patient reached their specific healing time (5, 6, and 9 weeks for T1, T2, and T3, respectively).

Specimens Harvested

When each patient reached the end of their healing time, the same well-trained surgeon (Q.S.) harvested a bone core biopsy sample from the implant site. Full-thickness flap elevation was needed in order to expose the donor site completely. The centers of the trephine cores and implant osteotomies were located at least 5.0 mm from the proximal surfaces of the adjacent teeth. Under irrigation with a saline solution, biopsy samples were harvested using a cylinder trephine bur (2-mm inner diameter, 16-mm length) by means of transcervical methods. Based on each patient’s pretreatment CBCT scan, the drilling depth was determined to penetrate the preexisting alveolar bone though the crestal bone into at least 4 mm of grafted bone. In this way, adequate bone volume consisting of both autogenous bone and grafted regions could be guaranteed.

The biopsy samples must be removed carefully from the trephine bur. After harvesting the samples, a drill sequence was then followed to prepare the osteotomy for simultaneous implant placement. Implants with a 4.8-mm diameter, 10.0-mm length, 1.8-mm supracrestal collar, and 6.5-mm restorative platform (Standard Plus WN, Straumann) were then inserted. A total of 10 bone core samples were retrieved, and 10 implants were placed.

Histologic Preparation and Analysis

Specimens were fixed in 4% paraformaldehyde/phosphate-buffered saline, embedded in paraffins, and decalcified at room temperature with 0.5 M ethylenediaminetetraacetic acid (EDTA) at 5.2 pH for 48 hours (short time, n = 5 specimens) and 1 week (long time, n = 5 specimens). Using a microtome (CRM-440, Sakura Seiki), specimens were then sectioned to 5-μm thickness along the full midline longitudinal length of the core. Immunohistochemical staining (Santa Cruz Biotechnology) of BMP-2 and RUNX2 were performed on paraffin-embedded sections. After deparaffinization of sections in xylene and rehydration with graded alcohol, nonspecific sites were blocked with 0.3% H2O2 for 20 minutes at room temperature. Sections were incubated with primary antibody against BMP-2 (sc-6895, 1:50) and RUNX2 (sc-8566, 1:100) overnight at 4°C. To detect the primary antibody, an immunohistochemical detection system (REAL EnVision, Dako) was added according to the manufacturer’s instructions. Finally, counterstaining with Mayer’s hematoxylin was performed. Control experiments included omission of primary antibody as a negative control, and tissues known to express the protein of interest as a positive control. The slices were analyzed under light microscopy (BX53, Olympus) from ×100 to ×200 magnification. The numbers of BMP-2–positive and RUNX2–positive areas were determined by analyzing ten random ×100 fields of each specimen. Quantitative histomorphometric analysis was performed with respect to BMP-2 and RUNX2 among the five groups.

For Masson and Von Gieson staining, representative ×100 images were selected to show the histomorphometric details, while for hematoxylin and eosin (h&e) staining, ×200 images were selected.
The statistical descriptions of BMP-2 and RUNX2 density were presented as mean and SD. Analysis of variance was used to compare means among the groups. Tukey honest significant difference method was used for pairwise comparison. The trends of BMP-2 and RUNX2 over time were fitted by quadratic regression. All statistical analyses were performed by JMP 14.0 (SAS Institute), and $P < .05$ was considered statistically significant.

### Results

**Clinical Examination**

For the five patients treated in the present study, sinus grafting was performed at maxillary molars sites. There were no membrane perforations in any treated patients. Two bone cores were collected per patient because of continuous dentition defect. No inflammation was found, and all soft tissue surrounding the incisions exhibited rapid maturation and had a healthier presentation than the control (Ctl) group. CBCT scans showed intact dome augmentation above the sinus floor, which encompassed the inserted implants with enough grafting materials.

**Histomorphometric Analysis**

h&e staining

Based on the histologic analysis in Fig 1, the areas of newly formed bone were significantly larger in the HBO groups (T1, T2, and T3) compared to the Ctl group. In the Ctl group, with natural healing for 6 weeks, no obvious newly formed bone was observed, and only limited provisional matrix were aligned along the substitute chips. Detached bone-substitute chips were observed, surrounded by fibrous capsular tissue.

However, in the groups with HBO treatment and increased healing times (T1, T2, and T3), newly formed bone was distinguished clearly, and a greater volume of old bone was presented, including Haversian lamella. In the TM group, the complete network structure made from newly formed bone was seen.

Von Gieson staining and Masson staining

Von Gieson staining is shown in Fig 2, and Masson staining is shown in Fig 3. In the Ctl group, almost no new bone was observed and a large amount of provisional matrix surrounded the Bio-Oss chips (Figs 2a and 3a). Compared to the Ctl group, as the healing time increased...
for HBO treatment (subsequent T1, T2, and T3 groups), newly formed bone gradually increased, and bone deposition and new bone mineralization were higher. More lamellar bone was observed in T2 and T3 compared to T1. The relatively mature bone was presented in T3 (Figs 2d and 3d), where Havel’s system was formed by thickened trabecular bone, and abundant blood vessels occurred. In the TM group, mature bone was connected with each other and encompassed the Bio-Oss chips.

Fig 2 Histologic images (von Gieson stain, × 100 magnification, scale: 200 µm) from the (a) Ctl, (b) T1, (c) T2, (d) T3, and (e) TM groups. Provisional matrix (yellow arrow), Bio-Oss chips (yellow triangle), newly formed bone (yellow star), the Haversian system (white arrow), and neovascularization (red arrow) could be seen in the samples.

Fig 3 Histologic images (Masson stain, × 100 magnification, scale: 200 µm) from the (a) Ctl, (b) T1, (c) T2, (d) T3, and (e) TM groups. Provisional matrix (yellow arrow), Bio-Oss chips (yellow triangle), newly formed bone (yellow star), the Haversian system (white arrow), and neovascularization (red arrow) could be seen in the samples.
**BMP-2 and RUNX2 Expression**

BMP-2 was expressed in osteoblasts, osteoprogenitor cells, and some connective tissues (Fig 4). Representative immunohistochemical views showed BMP-2 mainly in vital bone and connective tissue among the five different groups. HBO treatment increased BMP-2 expression at the edge of vital bone and in the connective tissues (Figs 4b to 4d), shown by the brown-colored flecks.

Representative immunohistochemical views from RUNX2 expression staining showed a similar pattern to BMP-2 (Fig 5); it was involved in connective tissue and at the edge of vital bone. HBO treatment (T1, T2, T3 groups) increased RUNX2 expression in the nucleus compared with Ctl. Without HBO treatment (TM) after 13 months, optical density of RUNX2 (TM) had a trend similar to Ctl. In Figs 5c and 5d, a yellow ring identifies more brown-colored, RUNX2-positive areas of the nucleus compared to Ctl, T1, and TM.

Table 1 shows the different levels of BMP-2 and RUNX2 optical densities. There were statistically significant differences in BMP-2 levels among all groups, apart from T2/T3 (P = .0594) and T2/TM (P = .4020). Even though there are no statistically significant differences between T2 and T3 groups, T3 still presented the highest mean optical density (532.45 ± 25.23 optical density) in all groups. When analyzing the RUNX2 optical densities, statistically significant differences were seen among all groups, apart from T2/T3 (P = .1455) and Ctl/TM (P = .7492). In HBO groups, HBO significantly increased the RUNX2 optical densities compared with non-HBO groups (Ctl and TM). Both BMP-2 and RUNX2 showed quadratic trends, which initially presented an upward trend, followed by a downward trend depending on the T1, T2, and T3 groups in Figs 6 and 7.

**Discussion**

Methods of reconstructing bone in areas of maxillary resorption and sinus pneumatization continue to evolve. Maxillary sinus augmentation with grafting materials can increase the bone height in the posterior maxilla. A large number of studies have demonstrated that elevation of the maxillary sinus floor is an effective and common technique to improve implant survival rates. A large number of studies have demonstrated that elevation of the maxillary sinus floor is an effective and common technique to improve implant survival rates.
bone volume, facilitating implant placement in critical defects.\textsuperscript{13} Based on histologic assessments, this particular bone graft (Bio-Oss) has been proven and widely recognized to be a suitable bone substitute for maxillary sinus elevations.\textsuperscript{14–16} Lacking osteoinductive properties as xenografts, time is required for sufficient bone healing after grafting. In the present authors’ opinion, it is of great significance to explore a more effective method to promote rapid bone healing. Only in this way will it be beneficial in reducing patient healing waiting times, shortening the total treatment time, and restoring the patient’s masticatory function as soon as possible.

A considerable number of previous studies have shown that osteogenic activity could be enhanced as a result of HBO treatment.\textsuperscript{7,17} However, most results regarding HBO

**Table 1 BMP-2 and RUNX2 Optical Densities Among Each Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2</th>
<th>RUNX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td>141.67 ± 70.54\textsuperscript{abdg}</td>
<td>50.83 ± 3.93\textsuperscript{abk}</td>
</tr>
<tr>
<td>T1</td>
<td>273.89 ± 24.71\textsuperscript{cegh}</td>
<td>95.84 ± 18.18\textsuperscript{ehkl}</td>
</tr>
<tr>
<td>T2</td>
<td>449.3 ± 82.42\textsuperscript{be}</td>
<td>154.59 ± 24.37\textsuperscript{bei}</td>
</tr>
<tr>
<td>T3</td>
<td>532.45 ± 25.23\textsuperscript{cf}</td>
<td>133.39 ± 29.82\textsuperscript{ajl}</td>
</tr>
<tr>
<td>TM</td>
<td>396.08 ± 97.6\textsuperscript{dfh}</td>
<td>61.64 ± 13.76\textsuperscript{hi}</td>
</tr>
</tbody>
</table>

Data are displayed as mean values ± SD. Each group comprised 10 biopsy samples for analysis. Ctl = 6 weeks of natural healing; T1 = 5-week healing with hyperbaric oxygen (HBO); T2 = 6-week healing with HBO; T3 = 9-week healing with HBO; TM = 13 months of natural healing.

\*\*P < .01.
\*\*\*P < .0001.

*Significant difference (***) between T3 and Ctl.
*Significant difference (***) between T2 and Ctl.
*Significant difference (***) between T3 and T1.
*Significant difference (***) between TM and Ctl.
*Significant difference (***) between T2 and T1.
*Significant difference (***) between T3 and TM.
*Significant difference (***) between T1 and Ctl.
*Significant difference (**) between TM and T1.
*Significant difference (**) between T2 and TM.
*Significant difference (**) between T3 and TM.
*Significant difference (**) between T3 and T1.
*Significant difference (**) between T1 and Ctl.
therapy are presented in in vitro studies. Animal experiments have confirmed that HBO is beneficial for the immediate implants placed into autogenous bone grafts. As far as the present authors are aware, the present clinical in vivo study is the first time to explore the effect of HBO on osteogenesis in human maxillary sinus lateral augmentation.

To investigate the bone healing process, histomorphometric analysis is an extremely efficient method and is the first step of identifying bone graft substitution via replacement with vital bone during the bone healing process. Herein, morphologic analysis and immunohistochemical evaluation were used to compare the expression of two important osteogenesis-related factors, BMP-2 and RUNX2, in order to explore the ideal window of time for HBO in the procedure. It is an innovative attempt to clinically prove that HBO is an effective method and can improve osteogenesis in the maxillary sinus lateral grafting.

Studies have shown that HBO enhances regenerative capabilities in mammals and that it is a safe, noninvasive modality. One study indicated that HBO could be used to increase the quality and the quantity of the newly formed bone. The Wnt3a/β-catenin pathway is an important signaling pathway in osteogenesis, and current studies have demonstrated that this pathway not only promoted osteogenesis but also inhibited adipocyte differentiation. HBO can promote the proliferation of neural stem cells by promoting the expression of the Wnt-3 protein. Lin et al found that HBO can stimulate BMP-2 production through Wnt3a/β-catenin signal transduction pathway, which implies that HBO is an effective therapeutic approach to promote new bone formation in the clinic. Kloen et al showed that BMP-2 expression was significantly reduced in nonhealing sites of human chondrocytes compared with fractured callus, suggesting that the osteogenesis effects of HBO are likely mediated via BMP signal pathways.

For the first time, the results of the present in vivo study showed that the BMP-2 expression intensities from maxillary sinus elevation with grafts were increased by early HBO stimulation. The results showed that BMP-2 expression intensity had a linear, corresponding relationship with the use of HBO to weeks 5 (T1), 6 (T2), and 9 (T3). BMP-2 expression intensity increased gradually and showed an apparent time-dependent trend according to amount of time HBO was used. The expression intensity peaked at T3, and there was no significant difference in the expression intensity between T2 and T3 (P = .0594). Although there was no statistical difference between T2 and TM
RUNX2 has a quadratic relationship to HBO until 6 to 9 weeks after surgery. Neither BMP-2 nor RUNX2 expression occurred 9 weeks after surgery, while RUNX2 reached the strongest value 6 weeks after surgery. Neither BMP-2 nor RUNX2 expression intensities showed statistically significant differences between T2 and T3. Therefore, it can be deduced that HBO-assisted healing of maxillary sinus lateral augmentation with Bio-Oss can be preferred, compared to 13 months of natural healing, due to the higher expression intensities of both BMP-2 and RUNX2, which are very beneficial in osteogenesis. The present authors suggest continued use of HBO until weeks 6 to 9 weeks after surgery.

It has been found that in vitro matrix mineralization requires additional tissue-specific combined factors to supply the activity of RUNX2, and RUNX2 is critical to multiple osteoblast-specific activities. Bioinformatics analysis showed that RUNX2 played an important role in chondrocyte maturation and osteoblast differentiation, and it also promoted the formation of axons and hematopoietic cells during bone development. It can be inferred that the human body is an organic whole, in which BMP-2 and RUNX2 are interrelated, interdependent, and mutually reinforced. Both of them can promote osteogenic function, which may explain the increased expression intensities of the two factors together in the present study. Such results are consistent with the previous study that BMP-2 and RUNX2 played an important role in promoting bone formation. Up to now, it is still unknown whether HBO could inhibit the osteoclast activities, and this should be further investigated in the future.

As far as the present authors are aware, this in vivo clinical study is the first report to explore the effect of HBO on osteogenesis in maxillary sinus lateral augmentation for human beings. Due to strict contraindications and indications of HBO in clinical practice, only five proper patients were included in the study. Additionally, factors such as patient age, sex, and other systemic diseases might induce limitations and imprecision of the outcomes. However, this study fostered a new idea to attempt the correct usage of HBO in maxillary sinus grafting in the future. Further in vivo and in vitro studies regarding the precise signal pathways are needed to be examined. The present authors are conducting a randomized controlled study based on gene sequencing of osteoblasts in patients with sudden deafness who prefer to receive HBO at the early stage. Clear
osteogenesis mechanism by HBO will be drawn.

Conclusions

Within the limitations of this study, the results indicate that early stimulation of HBO and maintaining its use until 6 to 9 weeks after maxillary sinus lateral augmentation with xenograft seemed to be the best time frame to yield outcomes beneficial to bone healing efficiency.

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